

Decreased DNA Repair Rates and Protection From Heat Induced Apoptosis Mediated by Electromagnetic Field Exposure

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In this study, we demonstrate that electromagnetic field (EMF) exposure results in protection from heat induced apoptosis in human cancer cell lines in a time dependent manner. Apoptosis protection was determined by growing HL-60, HL-60R, and Raji cell lines in a 0.15 mT 60 Hz sinusoidal EMF for time periods between 4 and 24 h. After induction of apoptosis, cells were analyzed by the neutral comet assay to determine the percentage of apoptotic cells. To discover the duration of this protection, cells were grown in the EMF for 24 h and then removed for 24 to 48 h before heat shock and neutral comet assays were performed. Our results demonstrate that EMF exposure offers significant protection from apoptosis ($P < .0001$ for HL-60 and HL-60R, $P < .005$ for Raji) after 12 h of exposure and that protection can last up to 48 h after removal from the EMF. In this study we further demonstrate the effect of the EMF on DNA repair rates. DNA repair data were gathered by exposing the same cell lines to the EMF for 24 h before damaging the exposed cells and non-exposed cells with H₂O₂. Cells were allowed to repair for time periods between 0 and 15 min before analysis using the alkaline comet assay. Results showed that EMF exposure significantly decreased DNA repair rates in HL-60 and HL-60R cell lines ($P < .001$ and $P < .01$ respectively), but not in the Raji cell line. Importantly, our apoptosis results show that a minimal time exposure to an EMF is needed before observed effects. This may explain previous studies showing no change in apoptosis susceptibility and repair rates when treatments and EMF exposure were administered concurrently. More research is necessary, however, before data from this in vitro study can be applied to in vivo systems. *Bioelectromagnetics* 23:106–112, 2002. © 2002 Wiley-Liss, Inc.

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INTRODUCTION

Contemporary society is developing an increasing dependency on electrical devices and appliances. This increased dependency has caused concern over the possible negative health effects of the electromagnetic fields (EMF) generated by such devices. These concerns have become more pronounced since a study in 1979 demonstrated a correlation between an increased risk of Acute Lymphocytic Leukemia (ALL) and 60 Hz EMF exposure [Wertheimer and Leeper, 1979]. Since the publication of this study, a controversy has raged concerning the connection of EMF exposure and cancer. Currently no conclusive evidence exists linking in vivo EMF exposure and cancer. However, several in vitro effects of EMF exposure have been noted. These effects include increases in DNA synthesis [Cossarizza et al., 1989a], Ca²⁺ signaling [Lindstrom et al., 1993; Korzhseptsova et al., 1995; Walleczek and Liburdy, 1990; Walleczek et al., 1996], RNA synthesis [Phillips and McChesney,

1991], and gene expression [Goodman and Henderson, 1986], along with changes in cell proliferation [Liburdy et al., 1993; Cain et al., 1993; Harland and Liburdy, 1997] and stimulation of SRC family kinases [Uckun et al., 1995]. (See Lacy-Hulbert et al. [1998] for a more comprehensive review of EMF research to date).

Recent studies investigating changes in susceptibility to apoptosis with regard to EMF exposure have reported both decreased [Fanelli et al., 1999] and increased susceptibility [Flipo et al., 1998; Ismael et al., 1998]. Interestingly, previous studies involving DNA repair and EMF exposure have reported no effect [Cossarizza et al., 1989a; Frazier et al., 1990; Cantoni

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et al., 1995, 1996]. However, these DNA repair studies allowed repair to occur concurrent with EMF exposure. We hypothesized that these studies might be obscuring the EMF's effect if a minimal time period of EMF exposure were needed before changes in cell physiology occurred. This hypothesis seemed especially attractive in light of a recent study demonstrating that 2 h exposure to a 1 mT EMF results in upregulation and differential phosphorylation of heat shock proteins (Hsps) [Pipkin et al., 1999], which have been shown to mediate a decrease in apoptosis susceptibility and correlate with a decrease in DNA repair rates [Samali and Cotter, 1996; Schmidt-Rose et al., 1999].

In this paper, we present data that demonstrated a decrease in apoptosis susceptibility induced by heat shock and a decrease in DNA repair rates following oxidative damage in two human cancer cell lines (HL-60 and HL-60R) following 60 Hz EMF exposure. Interestingly, another human cancer cell line, Raji, only demonstrated a decrease in apoptosis susceptibility but not in DNA repair rates. However, all apoptosis data demonstrated a time dependency: a 12 h EMF exposure was needed to observe protection from apoptosis, which protection lasted up to 48 h after removal from the EMF. These results suggest that the effects of EMF exposure are dependent on the duration of exposure. They also suggest a possible mechanism by which EMF exposure may contribute to cancer formation. However, more research is necessary before applying the data from this *in vitro* study to *in vivo* systems.

MATERIALS AND METHODS

Cell culture. HL-60 cells (human promyelocytic leukemia, American Type Cell Culture (ATCC) CTL 240) were cultured in RPMI 1640 medium supplemented with 20% Fetal Bovine Serum (FBS) (Hyclone Laboratories, Inc.). Raji (human Burkitt lymphoma, ATCC CTL 86) and HL-60R cells (human promyelocytic leukemia with mutated retinoic acid receptor- α gene, obtained from Dr. B. K. Murray, Brigham Young University) were grown in RPMI 1640 medium supplemented with 10% FBS. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cells were kept in exponential growth throughout all experiments with viabilities $\geq 90\%$ as determined by cell counts and trypan blue exclusion.

Electromagnetic field exposure. Twenty-five milliliter cell culture flasks (Corning) containing HL-60, HL-60R and Raji cultures were exposed to a 0.15 mT EMF by being placed in the center of a Helmholtz coil. The coil apparatus had an internal diameter of 14 cm and a height of 10 cm that allowed

the placement of multiple or single flasks within the coil at one time. The flasks were placed so that they laid perpendicular to the direction of the generated magnetic field (vertical). The coils were stationed in the middle of a 37°C humidified incubator containing 5% CO₂. Non-exposed cultures (no EMF exposure) were incubated in one of four identical incubators without an EMF source. The Helmholtz coil and non-exposed cultures were randomly transferred between the different incubators throughout the course of experimentation to negate the possible differences in background EMF or temperature between individual incubators. Maintenance of all incubators at $37 \pm 0.8^\circ\text{C}$ was continually monitored using an HI 9040 microcomputer thermometer (Hanna Instruments).

Cells were maintained in the coil (or in a separate incubator for non-exposed cultures) for 4, 12 or 24 h. Following exposure to the EMF, the 4 and 12 h cultures were exposed directly to heat shock, while the 24 h cultures were taken to either heat shock, the DNA repair assay or transferred to a 37°C incubator for an additional 24, 36 or 48 h before heat shock (see Table 1 for an outline of time variables investigated).

Previous research [Pipkin et al., 1999; Chow and Tung, 2000] has shown the upregulation of Hsps after 1–2 h EMF exposure using field strengths 4–10 times greater than ours. In both studies, the 1–2 h EMF exposure at field strengths comparable to the strength of our system showed no effects. We hypothesized that a lower EMF strength would require a longer exposure time in order to see an effect. These time points were also chosen in order to provide a limited time course investigation of the biological effects of different EMF exposure and recovery times.

Cell proliferation assays. Cell proliferation was monitored for both EMF exposed and non-exposed cultures by using cell counts and [³H]-thymidine incorporation. Cell counts were obtained every 12 h for a 60 h period of time using a hemocytometer and cell counter (Coulter). Incorporation assays were prepared by seeding 24 wells of two different microwell plates with 200 μl of cells (5×10^5 cells/ml) and 50 μl of a [³H]-thymidine solution (1 μCi final concentration) per well. The [³H]-thymidine solution was prepared by diluting 50 μl of 1 mCi/ml 5'-[³H]-thymidine (6.7 Ci/mmol, Amersham) with 2.450 ml RPMI 1640 medium supplemented with 10% FBS. For each repetition, one microwell plate was placed in the EMF and the other in an identical incubator without an EMF. Following 24 h of exposure, both plates were removed. Twelve wells of EMF exposed cells were transferred to 12 empty wells of the non-exposed plate, and 12 wells of non-exposed cells were transferred to the EMF-exposed plate. By averaging the results from

TABLE 1. Time Variables Investigated Using HL-60 Cells. The Same Variables Were Investigated With Raji and HL-60R Cells

Time of exposure to 0.15 mT EMF (h)	Time following EMF exposure before heat shock (h)	Time following heat shock before neutral comet assay (h)	Time following EMF exposure before repair assay (h)
0 (non-exposed)	0	13	0
0 (non-exposed)	24	13	ND ^a
0 (non-exposed)	36	13	ND ^a
0 (non-exposed)	48	13	ND ^a
4	0	13	ND ^a
12	0	13	ND ^a
24	0	13	0
24	24	13	ND ^a
24	36	13	ND ^a
24	48	13	ND ^a

^a Not done.

the two plates, possible variances due to analysis of each individual plate and the transfer of cells between plates canceled out. Cells were harvested from the plates onto glass fiber filter paper (0.26 mm thickness, 1.0–1.5 μm pores, Whatman) using an Inotech cell harvester. The amount of incorporated [³H]-thymidine was analyzed using an automatic filter counting system (Inotech).

Heat shock. One milliliter of exponentially growing cells (approximately 5×10^5 cells) from each experimental group (non-exposed and EMF exposed) was immersed in a 43 °C water bath for 60 min. The temperature of the bath was maintained using a circulating water bath (PolyScience, model 8005) with a temperature stability of ± 0.05 °C. Negative control (non-heat shocked) apoptosis values were established by incubating cultures of both non-exposed and EMF exposed cultures at 37 °C for 60 min. Upon removal from the water baths, 1 ml of fresh medium was added to each sample before returning the samples to incubation at 37 °C in a 24 well-plate without an EMF. These conditions were used due to their ability to specifically induce apoptosis instead of necrosis, as established through earlier experiments [O'Neill et al., 1998].

Neutral comet assay. Approximately 13 h \pm 30 min (the window of time determined by O'Neill et al. [1998] to demonstrate the maximum amount of apoptotic cells) was allowed to elapse between heat shock and performing neutral comet assays. Neutral comet assays, which can accurately discriminate between apoptotic and necrotic cell death [Fairbairn and O'Neill, 1996], were performed and analyzed according to the protocol described by O'Neill et al. [1998]. The percentage of apoptotic cells for each experimental group was then determined from the results of this assay.

Repair assay. The repair assay is a modification of the alkaline comet assay described by Fairbairn et al.

[1993, 1994]. Cells from both non-exposed cultures and cultures exposed to a 0.15 mT EMF for 24 h were suspended in Hank's balanced salt solution (HBSS) and damaged with H₂O₂ (1 mM final concentration, Fisher Scientific). Samples were centrifuged and resuspended in a low melting point agarose solution (0.75% final concentration, Sigma-Aldrich Co.) and immediately layered on custom frosted slides [Smith and O'Neill, 1998]. Slides were transferred to either a 4 °C lysis solution (described by Fairbairn et al. [1993] or to RPMI 1640 medium with 10% FBS at 37 °C for 7.5 or 15 min. Slides that incubated in the RPMI solution were immediately transferred to the 4 °C lysis solution following the specified RPMI incubation time. After addition of the last set of slides to the lysis solution, all slides were further incubated in the lysis solution for an additional 45 min. The alkaline comet assay was then performed as described by Fairbairn et al. [1993]).

Characterization of the electromagnetic field.

The sinusoidal electromagnetic field was generated by running a 120 W, 60 Hz current through a waveform generator that allowed for adjustment of a specific, constant output current. The waveform generator was provided by the Electrical Engineering Department, Brigham Young University (Provo, Utah). The output current (still at 60 Hz) was run through a Helmholtz coil and adjusted to produce an EMF with peak intensities of 0.15 ± 0.02 mT. The EMF strength was measured using a small coil at the end of a probe, placed in the center of the Helmholtz coil and connected to a Techtronics 465 oscilloscope. The oscilloscope allowed for recording of any current in the probe coil induced by the Helmholtz coil EMF. By knowing the number of loops in the probe coil and obtaining a reading of the current through the probe, the strength of the induced field, and therefore the Helmholtz produced EMF, was calculated. Using this technique it

was determined that the field strength was constant throughout the inside of the Helmholtz coil and that no significant EMF was detected outside of the coil in any of the incubators used. Maintenance of a constant current through the Helmholtz coil was continually monitored with a RMS voltmeter. The field strength measured at the conclusion of all experiments was within the standard deviation of the field strength measured at the onset of experimentation.

Statistical analysis. The percent of cells that underwent apoptosis was first calculated. A logit transformation was then performed on this data and used in a simple comparison between the non-exposed cultures and the EMF exposed cultures for each time point. For the DNA repair data, a log transformation was performed and analyzed by a repeated measures test using PROC MIXED in SAS.

RESULTS

Cell proliferation. Recent studies have reported a wide variety of conclusions concerning EMF exposure and proliferation rates of exposed cells. These diverse results include no change [Cain et al., 1993; Higashikubo et al., 1999; Loberg et al., 2000], increased cell proliferation [Harland and Liburdy, 1997; Wei et al., 2000; De Mattei et al., 1999] and decreased cell proliferation [Pacini et al., 1999; Stepanian et al., 2000]. These varied results may be due to the differences in exposure parameters as well as the type of cells investigated in each report. Our results from both cell counts and [³H]-thymidine incorporation demonstrated that there was no significant difference in proliferation rates between EMF exposed and non-exposed cells (data not shown). These results suggested that our apoptosis and repair data were not due to differences in cell cycle progression between EMF exposed and non-exposed samples.

Protection from heat induced apoptosis. Previous research demonstrates that EMF exposure can lead to the induction of Hsps [Pipkin et al., 1999; Blank and Goodman, 1999]. To further investigate the thermotolerant potential of EMF exposure, the heat sensitive [O'Neill et al., 1998] cell line, HL-60, was cultured in the presence of a 0.15 mT EMF for various time periods followed by heat shock (Table 1). A 4 h EMF exposure showed no significant difference in the percentage of apoptotic cells between EMF exposed and non-exposed cultures. However, 12 and 24 h exposures demonstrated protection against heat shock induced apoptosis, as seen by a significant ($P < 0.0001$) decrease in the percentage of apoptotic cells (Fig. 1). This reduction, as measured by the average of at least three repetitions, was almost two-fold: EMF exposed

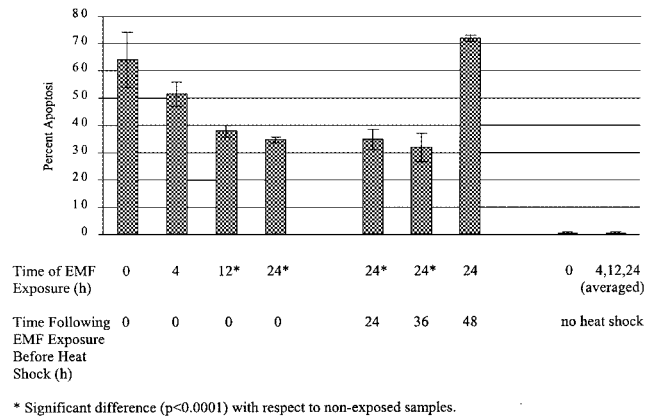


Fig. 1. EMF-induced time dependent protection from apoptosis in HL-60 cells.

cultures were approximately 35% apoptotic while non-exposed cultures were approximately 60% apoptotic. Non-exposed apoptotic values corresponded to previously established values for non-exposed cultures [O'Neill et al., 1998].

To investigate the duration of this thermotolerance, HL-60 cells were exposed to a 0.15 mT EMF for 24 h and then were maintained for 24, 36 or 48 h outside the field, followed by heat shock. Similar thermotolerance was observed when samples were allowed 24 or 36 h postexposure incubation ($P < 0.0001$), but this thermotolerance was absent in samples from the 48 h postexposure incubation (Fig. 1).

These experiments were repeated on another heat sensitive cell line, HL-60R, as well as a heat-insensitive cell line, Raji, to determine if protection from heat induced apoptosis was seen in other cell lines. A similar trend of a two-fold reduction in per-

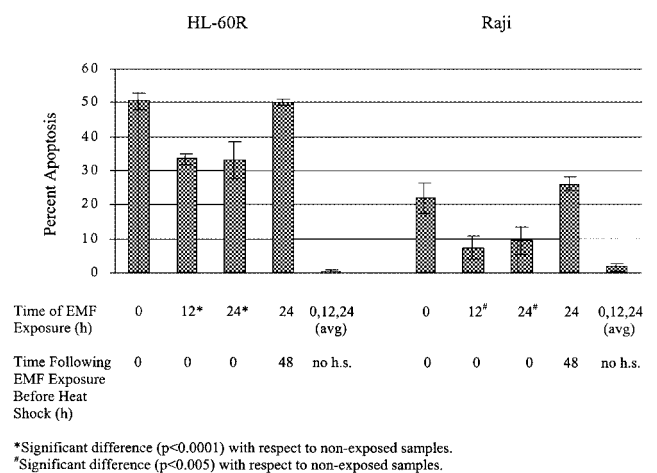


Fig. 2. EMF-induced time dependent protection from apoptosis in HL-60R and Raji cells.

centage of apoptotic cells was seen in both cell lines (Fig. 2). HL-60R cultures showed a decrease from approximately 55% apoptosis in control cultures to approximately 30% apoptosis in EMF-exposed cultures ($P < 0.0001$). Raji cultures decreased from approximately 20% apoptosis in non-exposed cultures to approximately 10% in EMF-exposed cultures ($P < 0.005$). Furthermore, the trend of thermotolerance induced by 12 h EMF exposure and abating by 48 h after removal from the EMF was observed in both cell lines.

Decreased DNA repair rates following EMF exposure. Previous data from studies of EMF exposure on DNA repair rates showed no effect [Cossarizza et al., 1989a, b; Frazier et al., 1990; Cantoni et al., 1995, 1996]. However, these earlier investigations exposed cultures to an EMF while concurrently inducing DNA damage or allowing for DNA repair. Since our data demonstrated a minimum time period before the induction of thermotolerance, we hypothesized that earlier methods used to assess an EMF's effect on DNA repair rates did not allow sufficient time for the EMF to alter cellular physiology. Therefore, we decided to investigate the effect of a 24 h EMF exposure prior to the administration of an oxidative damaging agent on DNA repair rates.

The alkaline comet assay is a sufficiently sensitive tool to monitor DNA repair [Meyers et al., 1993]. The parameter used to measure the amount of DNA damage in individual cells is tail moment. Tail moment is the product of the tail length and the fraction of DNA in the comet tail. The tail moment decreases as damaged DNA is repaired. The average tail moment of a population of cells is measured following allotted periods of time for repair. By comparing these values to the initial tail moment, relative rates of repair may be monitored.

In this investigation, the initial DNA damage induced by H_2O_2 varied slightly from repetition to repetition, but no significant difference existed between initial damage in EMF exposed and non-exposed cultures. We found that the rates of DNA repair for EMF exposed HL-60 and HL-60R cultures were significantly decreased ($P < 0.001$ and $P < 0.01$, respectively) when compared to non-exposed cultures (Figs. 3 and 4, respectively). Surprisingly, no difference in DNA repair rates was observed in EMF exposed Raji cultures when compared to non-exposed cultures (Fig. 5).

DISCUSSION

Past epidemiological studies demonstrate widely varying results concerning the connection between

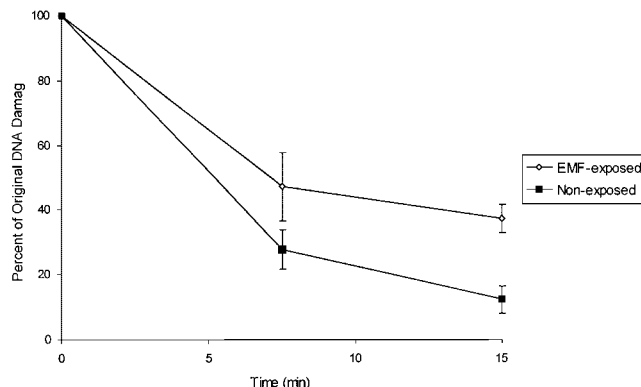


Fig. 3. Effect of 24 h EMF exposure on DNA repair rates in HL-60 cells.

EMF exposure and cancer. Therefore, evidence that elucidates the nature of this connection may be obtained first from in vitro studies. Our studies demonstrated that EMF exposure results in a time dependent decrease in susceptibility to heat induced apoptotic signaling for three human cancer cell lines as well as a time dependent decrease in DNA repair rates for two of these cell lines. Importantly, these results suggest a mechanism by which EMF exposure may influence tumor formation. The loss of sensitivity to apoptotic signaling is a common characteristic in the transformation of normal tissue to cancerous tissue [Jaattela, 1999]. In addition, the propagation of damaged DNA due to a decreased rate of DNA repair may also lead to oncogenic transformation. These two effects of EMF exposure may combine to further increase the probability of perpetuating DNA mutations that eventually lead to cancer.

Interestingly, two recently published reports have demonstrated a correlation between Hsps and altered DNA repair. One paper reported a decrease in DNA repair rates in an eukaryotic system following induc-

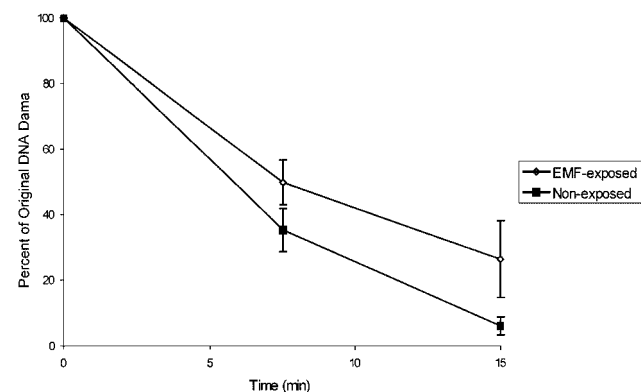


Fig. 4. Effect of 24 h EMF exposure on DNA repair rates in HL-60R cells.

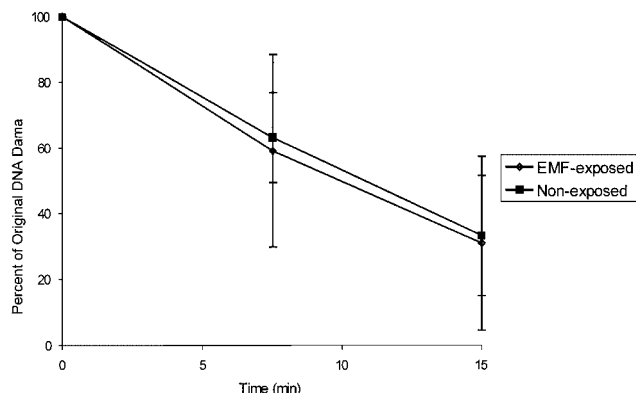


Fig. 5. Effect of 24 h EMF exposure on DNA repair rates in Raji cells.

tion of Hsps by mild hyperthermia [Schmidt-Rose et al., 1999]. The other paper reported an increase in DNA repair efficiency in *E. coli* [Chow and Tung, 2000] following both EMF exposure, which induced expression of Hsps, and transformation with Hsp expression vectors. To understand the differing results obtained by the Schmidt-Rose study, the Chow and Tung study, and this study, it is important to note that the Schmidt-Rose study did not directly confirm that the Hsps were the causative factor of the decreased DNA repair rates. Their results may have been due to other physiological changes in the cell induced by the hyperthermic treatment. In the Chow and Tung study, the results showed an increase in repair efficiency, not in repair rates. All three experiments investigated different variables and parameters of DNA repair.

Also of interest is the possible connection between Hsps, EMF exposure, and apoptosis that has been indirectly suggested by recent literature. The decrease in susceptibility to apoptotic signaling is a hallmark of Hsps [Samali and Cotter, 1996; Jaattela, 1999]. Hsps protect against apoptotic induction by signals such as TNF, anti-Fas, monocytes, caspase 3, serum starvation, oxidative stress, UV radiation, ceramide, etoposide, and others [Jaattela, 1999]. Previous research has established that EMF exposure results in the upregulation of Hsps after a 2 h exposure to a 1 mT EMF, but not after a 2 h exposure to a 0.1 mT EMF [Pipkin et al., 1999]. Our results, although not directly confirming the upregulation of Hsps in a 0.15 mT EMF, suggest that Hsps may be upregulated after 12 h exposure to a 0.15 mT EMF as evidenced by the protection from heat induced apoptosis that was observed.

This association between Hsps and EMF exposure is also suggestive of a "stressed state" within the cell that may be induced by EMF exposure and lead to the transcriptional upregulation or differential activa-

tion of Hsps, along with other intracellular molecules [Blank and Goodman, 1999]. This "stressed state" hypothesis could possibly explain why the heat insensitive Raji cell line did not demonstrate a decrease in DNA repair rates. Raji cells are less sensitive to stressful environmental conditions as evidenced by their lower susceptibility to heat induced apoptosis; therefore, these cells might also be less likely to respond to stressful conditions produced by EMF exposure.

It is also of particular interest that researchers have demonstrated that certain Hsps in several human cancers are overexpressed [Jaattela, 1999]. Previous findings concerning the relationship between Hsps, apoptosis, DNA repair and cancer combined with our results which demonstrated decreased apoptosis susceptibility and DNA repair rates in response to EMF exposure, suggest that exposure to an EMF may be involved in promoting tumor formation in in vivo systems. However, further research is needed to directly confirm the effect of EMF exposure on cancer formation as well as elucidating other physiological effects of EMF exposure.

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